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13. ABSTRACT ***(Maximum 200 Words)***

The purpose of this award is to study the transcriptional regulation of the BRCA2 breast cancer predisposition gene with the goal of identifying agents capable of modulating BRCA2 expression. Having previously mapped regions of the BRCA2 promoter that are involved in regulation of BRCA2 promoter function, we continued the project by examining the influence of a series of physiological and pharmacological agents on BRCA2 promoter function. Adriamycin, estrogen, serum starvation, forskolin, and tissue necrosis factor α either induced or repressed the promoter. In addition we demonstrated that the NFkB transcription factor can induce BRCA2 promoter function while adriamycin and p53 can repress the promoter. The observations that NFkB and p53 can regulate BRCA2 expression is expected to significantly impact our understanding of the pathways leading to cell death and to DNA damage repair. An improved understanding of the complex signals between the components of these pathways may facilitate design of novel therapeutic agents that can take advantage of these gene interactions.

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Eugene J. Couch 8/30/00

PI Signature Date

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2000 Annual Report DAMD17-97-1-7048

INTRODUCTION

The purpose of this award is to study the transcriptional regulation of the BRCA2 breast cancer predisposition gene with the goal of identifying agents capable of modulating BRCA2 expression. In this project we aim to test the effect of a variety of transcription factors, hormones, and environmental agents on BRCA2 expression and to define regions within the promoter that are responsive to these and other agents. By identifying agents that regulate the expression of BRCA2 it may be possible to design methods to induce or repress the expression of this tumor suppressor gene leading to enhanced DNA repair and/or apoptosis. Thus, agents that regulate BRCA2 expression may eventually be useful as forms of therapy for breast cancer. In previous years of this award we focused on defining the structure of the promoter and on identifying the regions of the promoter that regulate basal activity. In this report we describe the effect of a variety of agents on BRCA2 promoter function.

BODY

In this section we will describe the research that has been completed and then relate it to the specific tasks for the project rather than discussing each task independently.

USF regulates basal transcription from the BRCA2 promoter. Previously, we had shown that basal expression from the BRCA2 promoter is regulated by the USF transcription factor using deletion constructs and gel shift assays. To verify this effect a series of co-transfection studies were undertaken. Ectopic expression of USF1 or USF2 in Saos-2 and HMEC cells resulted in induction of the BRCA2 promoter as measured by a luciferase reporter construct and by increased levels of wildtype BRCA2 mRNA and protein. However, the level of induction was less than 2 fold in these cells. To evaluate whether USF must interact with a transactivating partner to induce the BRCA2 promoter, we ectopically expressed a USF-VP16 fusion construct in MCF7 cells. the USF-VP16 fusion protein induced a 4-fold increase in luciferase activity from the full length BRCA2 promoter and from the minimal promoter. In addition, mutations in the USF binding site ablated the increased luciferase activity. This suggests that USF interacts with other transactivating proteins to regulate basal transcription from the BRCA2 promoter. These data and those presented in the1999 report are reported in the manuscript attached in Appendix 1.

Induction of the BRCA2 promoter by NFκB. Following validation of the role of USF in regulation of BRCA2 basal transcription, we began to systematically map other transcription factor binding sites within the BRCA2 promoter which contribute to regulation of the promoter. Initially, we focused on the -144 to -58 region that was shown to induce basal transcription 3-fold. Sequence analysis of this region identified several putative transcription factor binding sites including an NFκB consensus binding site located at position -116 to -107 in the 8 kb BRCA2 promoter. To examine the role of NFκB in regulation of the BRCA2 promoter, the effect of overexpression of NFκB on luciferase activity was studied. Co-transfection of expression constructs of the p65 and p50 subunits of NFκB with the pGL3Prom reporter construct containing the wild type BRCA2 promoter resulted in significant induction of luciferase activity. Expression of

p65 alone and in combination with p50 increased activity 9 and 16-fold respectively. However, expression of p50 alone resulted in a small reduction in activity in comparison to a vector control.

In subsequent experiments we demonstrated that 1) NFkB dependent activation of the promoter requires the NFkB consensus binding site, 2) the p50 NFkB subunit binds to the NFkB consensus binding site in the promoter, 3) overexpression of NFkB subunits leads to in vivo induction of BRCA2, and 4) dominant negative and wildtype IκBα inhibit NFκB dependent induction of BRCA2. The studies demonstrating these effects are outlined in detail the manuscript attached in Appendix 1. These data clearly demonstrate that the NFkB transcription factor can induce BRCA2 expression by binding to the BRCA2 promoter. Thus these experiments address the aims stated in Task 2, 3, 7, 8, 9, and 10.

The effect of pharmacological and physiological agents on BRCA2 promoter activity. As outlined in Task 2 and 3, we proposed to study the ability of various agents to regulate BRCA2 expression. To address this task we have treated cells containing the BRCA2 promoter luciferase reporter construct with the agents listed in Table 1. A total of 1 X 10^5 MCF7 cells were plated in each well of a 6 well plate. After 24hrs these cells were exposed to the agents at the concentrations shown in Table 1. Alterations in luciferase activity were caused by adriamycin, estrogen, serum starvation, forskolin, and TNFα as shown in Table 1. UV-irradiation, gamma-irradiation, camptothecin, taxol, INFγ and vincristine appeared to have little effect. Previous publications have shown that estrogen upregulates the BRCA2 promoter indirectly through its general effect on the cell cycle. Serum starvation has been shown to downregulate BRCA2 expression possibly as a result of alignment of cells in G1 phase of the cell cycle where BRCA2 expression is absent. Effects of adriamycin, forskolin, and TNFα have not previously been detected.

The mechanism of BRCA2 inhibition by adriamycin. In response to these results we chose to examine the mechanism by which adriamycin (doxorubicin) influences BRCA2 promoter activity. Initially MCF7 cells were transfected with the BRCA2 promoter reporter construct and treated with 2.5-15mm adriamycin (ADR) for 1 hour. After 24 hours growth luciferase activity measurements showed that exposure to as little as 2.5mm ADR for 1 hour reduced basal activity from the BRCA2 promoter by 70%. Adriamycin is known to upregulate p53 expression. Thus we attempted to establish a link between induction of p53 and repression ofn the BRCA2 promoter in response to ADR. First we demonstrated that adriamycin could induce p53 expression by treating MCF7 cells transfected with a p53 dependent promoter (pGL13) with 5mm ADR in the presence and absence of wildtype and mutant p53. ADR treatment increased induction of the promoter by 30% while dominant negative p53 inhibited basla activity and ADR dependent activity. Next we demonstrated that the effect of ADR on the BRCA2 promoter is p53 dependent. MCF7 cells were transfected with the BRCA2 reporter along with either wildtype or dominant negative p53, and exposed to 5mm ADR. Wildtype p53 expression completely inhibited BRCA2 promoter activity in the presence and absence of ADR. In contrast, expression of mutant p53 resulted in a 30% increase in promoter activity. However, further treatment with ADR in combination with dn-p53 downregulated promoter activity by 50%, suggesting that mutant p53 inactivates the inhibitory function

of wildtype p53 and that ADR induces an excess of wildtype p53 which overcomes the effect of the mutant. Similar experiments in p53 null Saos-2 cells showed that ADR treatment had no effect on BRCA2 promoter activity suggesting that p53 is required for BRCA2 promoter inhibition. To further define the effect of p53 and ADR on the BRCA2 promoter we mapped the sites within the BRCA2 promoter that are responsive to ADR and p53 using a series of promoter deletion constructs that were described in the previous annual report. The results showed that both p53 and ADR regulate BRCA2 expression through the USF-1 binding site in the minimal BRCA2 promoter. When this site was deleted or mutated, BRCA2 promoter activity increased substantially, even in the presence of ADR or wildtype p53. Gel shift assays showed that treatment with ADR or expression of p53 reduced the binding of the USF-1 protein complex to the USF-1 site in the promoter. In addition, we were unable to demonstrate that p53 was a component of this complex suggesting that the p53 effect is indirect. Finally, we showed that teatment with ADR increased endogeneous levels of p53 and descreased endogeneous levels of BRCA2. Taken together these data suggest that ADR activates p53 which indirectly inhibits the ability of USF-1 to bind and activate the BRCA2 promoter. Thus these experiments address the aims stated in Task 2, 3, 7, 8, 9, and 10.

Interestingly, UV-irradiation, gamma-irradiation, camptothecin, and taxol are all known to induce p53 as a result of their DNA damaging activity. However, none of these agents had any effect on the BRCA2 promoter. This suggests that adriamycin affects the BRCA2 promoter through a specific p53 dependent pathway which is not influenced by these other agents. To begin to elucidate the signaling pathway we studied the role of p21 in the DAR response pathway. Expression of wildtype p21 in MCF7 cells or treatment of p21 null cells (kindly provided by Bert Vogelstein) with ADR had no effect on the response of the BRCA2 promoter to ADR and p53. Thus, the p53 and ADR dependent signaling pathway does not appear to involve p53 dependent induction of p21. In addition, it has previously been shown that UV-irradiation, gamma-irradiation, camptothecin, and vincristine all influence BRCA2 mRNA expression levels. However, in these direct promoter experiments no effect was detected. This suggests that the agents are causing increased stability of BRCA2 mRNA rather than induction of expression. We will attempt to prove this hypothesis in the next year.

Future directions. In the final year of this project we aim to:

1) Characterize the ADR and p53 dependent signaling pathway.
2) Characterize the estrogen effect on the BRCA2 promoter.
3) Characterize the effect of environmental estrogens such as Dioxin and 2,4,5-T on the BRCA2 promoter.
4) Characterize the –500 to –700bp region of the promoter that is associated with inhibition.
5) Characterize the 0 to +250bp region of the promoter that is associated with activation.
6) Investigate why certain agents cause increases in BRCA2 mRNA but do not activate the BRCA2 promoter.

Each of these aims further address the original aims and tasks of the proposal by identifying agents that regulate BRCA2 expression and specific cis-elements within the promoter that are responsible for this regulation.

KEY RESEARCH ACCOMPLISHMENTS

- Determined that NFkB transcription factor can upregulate BRCA2 expression
- Showed that adriamycin significantly downregulates BRCA2 expression
- Demonstrated that p53 downregulates BRCA2 expression
- Identified the USF-1 binding site as the mediator of the adriamycin and p53 effect on the BRCA2 promoter
- Determined that UV irradiation, γ-irradiation, camptothecin, taxol, vincristine, and INFγ have no effect of the BRCA2 promoter.
- Observed that Forskolin, TNFα, estrogen, and serum starvation alter BRCA2 promoter function

REPORTABLE OUTCOMES

One manuscript is in press in the Journal of Biological Chemistry:

Wu K, Jiang S-W, Muthusamy T, and Couch FJ. Induction of the BRCA2 Promoter by Nuclear Factor-κB. In press – J. Biol. Chem.

CONCLUSIONS

In summary, we have shown that the BRCA2 promoter can be upregulated by NFkB and downregulated by both adriamycin and p53. The observation that NFkB can upregulate BRCA2 is interesting because NFkB is a key regulator of cell proliferation and apoptosis. Thus, proliferative or apoptotic signals within the cell may upregulate BRCA2 in order to repair any DNA damage occuring during the proliferative phase, or to mediate apoptosis following cellular stress. However, the finding that adriamycin can repress BRCA2 is of greater importance. Adriamycin damages DNA, while BRCA2 has been associated with DNA damage repair. By repressing BRCA2 expression, adriamycin can prevent DNA damage repair while causing DNA damage. In addition, adriamycin induces p53 expression which can activate apoptotic signaling pathways in response to DNA damage. Thus, the combination of DNA damage, induction of p53 dependent apoptosis, and inhibition of BRCA2 dependent DNA repair by adriamycin suggests that this drug is particularly well suited as a chemotherapeutic agent. The observation that p53 can downregulate BRCA2 expression is also important because it may be possible to identify targets in this signaling pathway that will facilitate inhibition of DNA repair following treatment with DNA damaging chemotherapeutic agents.

APPENDICES

Table 1. Response of the BRCA2 promoter to various agents

Agent	**Concentration**	**% response**
Adriamycin	5 μM	80% reduction
Estrogen	6 μM	40% increase
Serum starvation		50% reduction
γ-irradiation	10 Gy	No change
UV-irradiation	10 J	No change
Camptothecin	5 μM	No change
Taxol	2.5 μM	No change
Vincristine	0.1 μM	No change
Forskolin	5 μM	30% increase
INF-γ	5 ng	No change
TNFα	10 ng	60% reduction

Induction of the BRCA2 Promoter by Nuclear Factor-κB

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Running Title: NFκB induces BRCA2 expression

SUMMARY

BRCA2 is a tumor suppressor gene that has been implicated in response to DNA damage, cell cycle control, and transcription. BRCA2 has been found to be overexpressed in many breast tumors, suggesting that altered expression of the BRCA2 gene may contribute to breast tumorigenesis. To determine how BRCA2 is overexpressed in tumors, we investigated the transcriptional regulation of the BRCA2 promoter. Deletion mapping of the BRCA2 promoter identified three regions associated with 3-fold activation or repression, and one USF binding site associated with 20 fold activation. Gel shift and cotransfection studies verified the role of USF in regulation of BRCA2 transcription. Analysis of the -144 to -59 region associated with 3-fold activation identified a putative NFκB binding site. Cotransfection of the p65 and p50 subunits of NFκB upregulated the BRCA2 promoter 16 fold in a luciferase reporter assay, while mutations in the binding site ablated the effect. Gel shift and supershift assays with anti p65 and p50 antibodies demonstrated that NFκB binds specifically to the NFκB site. In addition, ectopic expression of NFκB resulted in increased levels of endogeneous BRCA2 expression. Thus, NFκB and USF regulate BRCA2 expression through the BRCA2 promoter.

INTRODUCTION

BRCA2 is a tumor suppressor gene associated with familial predisposition to breast and ovarian cancer (1,2). Mutations in BRCA2 are thought to account for 20-35% of all inherited breast cancers and are associated with a 37-85% lifetime risk of developing cancer (3,4). The great majority of disease-associated mutations in BRCA2 result in truncation of the BRCA2 protein, suggesting that loss of function of BRCA2 results in tumor susceptibility. However, the mechanisms by which the BRCA2 protein suppresses tumor cell growth are largely unknown.

The BRCA2 gene encodes a 3418 amino acid nuclear protein (2,5), that has been implicated in the cellular response to DNA damage. BRCA2 interacts directly with RAD51, a protein involved in meiotic and mitotic recombination, DNA double-strand break repair, and chromosome segregation (6,7), through the BRC repeats and a C-terminal binding site. $BRCA2^{-/-}$ animals die as early embryos (8-11), and viable $BRCA2^{-/-}$ early mouse embryos are highly sensitive to γ-irradiation induced DNA damage (9). Moreover, cells expressing mutant BRCA2 are more sensitive to methyl methanesulfonate-induced DNA damage than cells expressing wildtype BRCA2 (12), and BRCA2 appears to be required for ionizing radiation-induced assembly of a RAD51 protein complex in vivo (13).

BRCA2 may be also involved in regulation of the cell cycle and genome instability. BRCA2 is expressed in a cell cycle dependent manner with peak expression in the S and G2 phases of the cell cycle. Low levels of expression are detected in G0, G1 and M phase (14). Cell cycle dependent expression has recently been associated with binding of the upstream stimulatory factor (USF) protein and Elf-1 transcription factor to the BRCA2 promoter (15). In addition, BRCA2 expression is elevated indirectly in response to the mitogenic activity of estrogen, which has been associated with progression of the cell cycle (16,17). Furthermore, recent studies of $BRCA2^{-/-}$ mouse embryo fibroblasts (MEFs) identified extensive chromosomal rearrangement,

centrosome amplification, and aneuploidy, consistent with abrogation of a mitotic checkpoint (18). Likewise, tumor cells expressing mutant BRCA2 have been shown to contain multiple chromosomal rearrangements (19). These data suggest that BRCA2 plays a key role in regulation of cell growth, and proliferation in many cell types.

Several studies have attempted to define a role for BRCA2 in development of sporadic breast cancer (20-25). Loss of heterozygosity of the BRCA2 locus has been detected in over 50% of sporadic breast tumors, suggesting a role for BRCA2 in sporadic breast cancer development (20-22). However, no somatic mutations of BRCA2 have been found in sporadic breast cancers (23,24). Also, the BRCA2 promoter is not inactivated by methylation in breast tumors (25). While no sequence alterations have been found in the BRCA2 gene in sporadic tumors, it remains possible that BRCA2 does contribute to sporadic breast cancer development, albeit not by inactivation of the BRCA2 protein through mutagenesis and methylation. One possible mechanism of BRCA2 involvement is through deregulated expression of the BRCA2 gene. Recently, it has been shown that BRCA2 is significantly overexpressed in many sporadic breast cancers (26). It is not known if this overexpression of BRCA2 is due to induction of the BRCA2 promoter or is a result of an increased number of cells in S phase of the cell cycle. However, when combining this observation with the known relevance of BRCA2 function to regulation of cell proliferation, it seems likely that expression of the BRCA2 gene is tightly regulated, and that altered expression of BRCA2 may contribute to breast cancer development.

To begin to assess the contribution of altered expression of BRCA2 to breast tumorigenesis, we investigated the transcriptional regulation of the BRCA2 promoter. Here we provide evidence for direct induction of the BRCA2 promoter through binding of the nuclear factor-κB (NFκB) transcription factor and we verify the role of USF in regulation of basal activity of the promoter.

EXPERIMENTAL PROCEDURES

Cell Culture - Human breast adenocarcinoma MCF-7 cells were obtained from American Type Culture Collection (ATCC), propagated in the Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% bovine calf serum (BCS) (HyClone) and maintained at 37°C with 5% CO_2. Cell culture reagents were obtained from Life Technologies Inc.

BRCA2 Reporter Constructs - A BAC clone (B489G) containing the 5' end of the BRCA2 gene was isolated from a BAC library (27) using a polymerase chain reaction (PCR) generated hybridization probe consisting of bases 72-560 of the BRCA2 cDNA. B489G DNA was digested with SacI and PstI enzymes and the resulting fragments were subcloned into the pGL3 basic vector containing a firefly luciferase reporter gene (Promega) and plated. Colonies containing the 5' end of the BRCA2 gene were identified by hybridization with the 72-560 bp cDNA probe. Plasmid DNA from positive colonies was prepared and sequenced using vector specific primers. Sequences were then matched against the complete genomic sequence of this region in Genbank. A clone with an 8 kb insert (pGL3Prom) was found to include 4.3 kb of sequence upstream of the putative BRCA2 transcription start site, and 3.7 kb downstream of the transcription start site including exons 1, 2, and 3 of BRCA2. The entire 8 kb insert was then sequenced by the Molecular Biology Core of the Mayo Clinic.

Deletion Mutants of the BRCA2 Promoter - A series of deletion mutants (Figure 1 and 2) of the BRCA2 promoter were generated by restriction enzyme digestion with a variety of restriction enzymes followed by religation, and also by direct PCR amplification. The Del-1 construct was generated by digesting the pGL3Prom construct with HindIII and PstI and religating the

pGL3Prom plasmid. Del-2 resulted from religation following digestion with MluI and PstI. Del-9 was generated by subcloning a 1249 bp fragment of pGL3Prom, resulting from KpnI and MluI digestion, into the pGL3 basic promoter. Del-2 was then digested by combinations of SacI with NdeI, HindIII, EcoRI, and BbrPI, the linearized plasmids were blunt-ended with Klenow enzyme (New England BioLabs), and religated to form Del-3, Del-4, Del-5, and Del-16 respectively.

Additional deletion mutants were constructed by PCR based strategies. PCR primers were designed containing a SacI or KpnI site in 5' forward primers and a PstI site in 3' reverse primers. The 5' forward primers used in the constructs Del-6-8, and Del-10-15 were: Del-6 (-897), 5'-TGGGTGTGGGAGCTCATGCCTGTAATCC-3'; Del-7 (-796), 5'-AAACCCCGAGCTCTACTTAAAAATGCA-3'; Del-8 (-678), 5'-GGAAGTTGCGGTGAGCTCAGATTGCG-3'; Del-10 (-515), 5'-ACGGGCTCGGAGCTCTTGAACAC-3'; Del-11 (-422), 5'-ACTAAGTGAGCTCATCCA-CAACC-3'; Del-12 (-310), 5'-AAGGTATTTCAGAGCTCCCAGG-3'; Del-13 (-236), 5'-GACTTGGAGCTCAGGCATAGG-3'; Del-14 (-144), 5'-TATTCGAGCTCAGA-TACTGACGG-3'; Del-15 (-58), 5'-CCAGGCCTGAGCTCCGGGTG-3'; The single 3' reverse primer was 5'-AGCCCGGGCCTGCAGGCGTGGCTAG-3' which contains a PstI site. The 3' reverse primers used for Del-17 and Del-18 were: Del-17 (0), 5'-TTCAGAAGCTCGCTGCAGAAGCCCGCGCTGG-3' and Del-18 (+110), 5'-TCTGTCCCCTGCAGGCTTCTCC-3'. The single 5' forward primer was 5'-TGCGGAG-CAAGGGAGCTCACACTTCATGAGC-3' which contains a KpnI site. PCR products were generated using *pfu* DNA polymerase (Stratagene), and 30 ng of Del-2 as template DNA. PCR conditions were as follows: 1 cycle for 2 min at 95 °C; 20 cycles at 95 °C for 30 sec, 55 °C for 1 min and 68 °C for 4 min; and 1 cycle at 68 °C for 10 min. The PCR products were digested with either SacI and PstI or KpnI and PstI restriction enzymes and ligated into pGL3 basic vectors.

Deletion mutants generated with PCR were sequenced using an automated DNA sequencer to monitor for PCR-associated nucleotide incorporation errors.

Point Mutants of the BRCA2 Promoter - Site-directed mutagenesis of the Del-15 construct was performed using the QuikChange site-directed mutagenesis kit (Stratagene) to prepare constructs containing mutations in predicted cis-elements within the promoter. Specifically, mutations were introduced into putative DNA binding sites for the ATF, USF, MLTF, and c-myc transcription factors (Figure 2). Mutations were confirmed by DNA sequencing.

Luciferase Reporter Assays - Plasmid DNA for transient transfection was isolated using the plasmid maxi kit (Qiagen). MCF-7 cells were plated at a density of $1x10^5$ cells / per well of 6-well plates and grown in DMEM with 10% BCS overnight prior to transfection. All transfections were carried out using Fugene-6 (Boehringer Mannheim Biochemicals) according to the manufacturer's instructions. A total of 2 μg of BRCA2 promoter construct and 0.1 μg of pRL-CMV Renilla luciferase vector (Promega) with 4 μl of Fugene-6 was used for each transfection. The pRL-TK Renilla luciferase activity was used to control for transfection efficiency. Each transfection experiment was performed in duplicate and repeated a minimum of three times. For co-transfection experiments, cells received 0.5 μg of BRCA2 promoter construct, 0.1 μg of pRL-TK Renilla luciferase vector, and 0.5 μg of the indicated expression plasmids and carrier DNA. Expression plasmids included pCMV-USF, pCMV-USF-VP16, pCMV-VP16, pCMV, pcDNA3.1-p65, pcDNA3.1-p50, pcDNA3.1, pCMV-CREB, pCMV-Myc, and pCMV-Max. Firefly luciferase and renilla luciferase assays were performed using the Dual-Luciferase Reporter Assay System (Promega). Approximately 48 hours after transfection, cells were washed twice with 1x PBS

and harvested with 600 µl of passive lysis buffer (Promega). Cell lysates were cleared by centrifugation and 5 µl was added to 100 µl of firefly luciferase substrate, and light units were measured in a luminometer. Renilla luciferase activities were measured in the same tube after addition of 100 µl of Stop and Glo reagent.

Electrophoretic Mobility Shift Assays (EMSAs) - Double strand oligonucleotides generated from the single strand oligonucleotides listed in Table 1 and 2 were used as EMSA probes. The upper strand (sense) oligonucleotide (30ng) was 5' end labeled using polynucleotide kinase with γ-^{32}P-dATP (Amersham Life Sciences). After the labeling reaction, two-fold excess of lower strand (antisense) oligonucleotide was annealed to the upper strand. Double strand DNA probes were purified from the reaction mixture using a Bio-Gel P-100 column (Bio-Rad). Whole cell extracts were isolated from cultured MCF-7 cells. DNA-protein binding was performed in 0.5X Dignam buffer D (20 mM HEPES (pH7.9), 100 mM KCl, 20% glycerol, 0.2 mM EDTA) supplemented with 0.5 mM PMSF, 0.5 mM DTT, 10mM $MgCl_2$ and 100 µg/ml poly (dI-dC). Binding reactions were initiated by addition of 30,000 cpm DNA probe in TE buffer (10mM TRIS.HCl (pH7.5), 1mM EDTA) to 5 to 10 µl of whole cell extracts. Electrophoresis was performed in acrylamide gels, gels were dried and exposed to film for 16 to 48 hours.

Competition experiments were carried out in the same way as described above except that increasing amount of double stranded wild type oligonucleotide were mixed with 30,000 cpm of M-1 or M-2 probes (Table 1) and added to the binding reaction. For optimized antibody mediated super shift experiments, increased DNA probe (60,000 cpm) and decreased whole cell extracts (5 µl) were applied. The binding reaction included 1 to 4 µl of antibodies against ATF2

(Santa Cruz Biotech), c-myc (Santa Cruz Biotech) or USF-1 (kindly provided by Dr. Michele Sawadogo, M.D. Anderson Cancer Center).

DNA binding assays for nuclear factor-κB (NFκB) were also performed using EMSAs. Whole cell extracts were prepared from MCF-7 cells 48 hours after transfection with pcDNA 3.1 or NFκB p65 and p50 subunit expression constructs. Components of NFκB proteins were identified by supershift assay using antibodies against p50 and p65 (Santa Cruz Biotech).

Western Blotting - Forty eight hours after transfection with pcDNA 3.1 or p50 and p65 expression constructs, MCF-7 cells were washed with 1x PBS, and cell lysates were prepared with RIPA buffer containing COMPLETE proteinase inhibitor cocktail (Boehringer Mannheim). Equal amounts of protein lysate from each transfection were subjected to electrophoresis, transferred to membrane, and probed with primary antibodies and alkaline phosphatase - conjugated secondary antibodies (Jackson ImmunoResearch Laboratories). Signals were developed by ECL detection system.

RNA Isolation and Northern Blotting - Total RNA was isolated from MCF-7 cells 48hr after transfection with pcDNA 3.1 or p50 and p65 expression constructs, or pcDNA3.1, dominant negative IκBα 32A/36A (dn-IκBα), NFκB p65, IκBα wildtype, p65 with dn-IκBα, or p65 with wildtype IκBα using TRIzol reagent (Life Technologies), according to the manufacturer's instructions. Total RNA samples (20 μg/lane) for pcDNA 3.1, p50, and p65 transfected cells were resolved on 0.8% agarose-formaldehyde gels and transferred to nylon membranes. The membranes were pre-hybridized at 62 °C for 1 hr in ExpressHyb Hybridization Solution (Clontech) and then hybridized for 1 hr in the same solution with α-^{32}P-ATP random labeled full-length hu-

man BRCA2 cDNA. After hybridization, the membrane were washed (3 times for 15 min each time at room temperature) with 2x SSC, 0.05% SDS, washed (3 times for 15 min each time at 62 °C) with 0.5x SSC, 0.1% SDS. Membranes were then exposed in a phosphorimager. Each membrane was also hybridized as described above with a GAPDH probe for normalization of mRNA levels.

Semi-quantitative RT-PCR analysis – 1 μg of total RNA from cells transfected with pcDNA3.1, dominant negative IκBα 32A/36A (dn-IκBα), NFκB p65, IκBα wildtype, p65 with dn-IκBα, or p65 with wildtype IκBα was used for preparation of cDNA with random hexamer primers and superscript II reverse transcriptase (Life Technologies). After treatment with DNAse, 2 μl from a total of 100 μl was used for semi-quantitative PCR with BRCA2 and GAPDH PCR primers. The sequences of forward (F) and reverse (R) PCR primers were as following: BRCA2, 5'-GCAGTGAAGAATGCAGCAGA-3' (F, within the exon 21 of human BRCA2), 5'-CAATACGCAACTTCCACACG-3' (R, within the exon 22 of human BRCA2); GAPDH, 5'-CAACTACATGGTTTACATGTTC-3' (F), 5'-GCCAGTGGACTCCACGAC-3' (R). Each PCR amplification was performed using Taq DNA polymerase (Promega) with both PCR primers for BRCA2 and GAPDH under the following conditions: 1 cycle for 2 min at 94 •C; 25 cycles at 94 •C for 30 sec, 54 •C for 30 sec and 72 •C for 30 sec; and 1 cycle at 72 •C for 10 min. The GAPDH product was used as a normalization control for the amount of cDNA in the PCR reactions. PCR products were subjected to electrophoresis using 6% polyacrylamide gels, stained by Sybr Green for one hour. Results were analyzed with a Molecular Dynamics phosphorimager system.

RESULTS

Identification of regulatory domains in the BRCA2 promoter - To analyze transcriptional regulation of the BRCA2 gene and to define functionally important cis-DNA elements in the 5'-flanking region of this gene, an 8 kb region of human genomic DNA containing the BRCA2 putative promoter was isolated from BAC clone B489G and subcloned into the pGL3 basic luciferase reporter vector. The 8 kb fragment contained 4.3 kb of sequence upstream of the putative transcription start site (2), and 3.7 kb downstream of the transcription start site as far as the 3' donor splice site of exon 3. This pGL3Prom and the pGL3 parent vector were transiently transfected into MCF-7 cells and luciferase activity was measured after 48 hours. All activities were normalized by activity measurements from cotransfected pRL-TK renilla luciferase vector. The pGL3Prom construct yielded 100 fold more luciferase activity than pGL3 suggesting that the pGL3Prom construct contained the BRCA2 promoter.

In order to identify the minimal BRCA2 promoter, a series of deletion constructs (Figure 1A) derived from pGL3Prom were generated as described above. Firefly luciferase expression was assayed following transient transfection of MCF-7 cells with these BRCA2 promoter constructs. The normalized luciferase activities for each deletion construct of the promoter relative to pGL3Prom activity are shown in Figure 1B. The results indicate that the BRCA2 promoter is regulated in a complex fashion. No change in activity was detected when comparing the Del-2 construct with pGL3Prom suggesting that the +668 to +3678 region has no influence on promoter activity. Deletion of the -4328 to -583 region caused a 2 fold increase in luciferase activity. Further deletion from -582 to -516 resulted in 2.5-fold activation of the promoter, while a 3-fold reduction in activity was detected following deletion of the -144 to -59 region. However, a 20 fold loss of luciferase activity was observed following deletion of a 40 bp region (-58 to -19),

suggesting that the region contains cis-elements that are critical for positive regulation of basal transcription activity in the BRCA2 promoter.

Analysis of the minimal BRCA2 promoter - In order to more accurately map the cis-element within the –58 to –19 bp region that regulates BRCA2 basal transcription, a further series of deletions were constructed using the Del-15 construct as a template (Figure 2A). Deletion of the -34 to –19 region (Del-16) resulted in a 12-fold reduction in luciferase activity in comparison to Del-19 (Figure 2A). Sequence analysis of a 20 bp region from –34 to –14 was carried out in an effort to identify putative transcription factor binding sites that might regulate BRCA2 basal transcription activity. The region was found to contain a tandem GCGTCACG repeat (Figure 2A) which encodes several predicted transcription factor binding sites including cis-elements for c-myc, USF, MLTF, and ATF transcription factors. A number of point mutations were introduced into the 16bp repeat sequence in the Del-15 reporter construct in an effort to identify the cis-element which was regulating basal transcription from the promoter. Substitution of nucleotides from repeat 1 resulted in a 4-fold loss in activity, while substitution of repeat 2 led to a 12-fold loss in luciferase activity (Figure 2A). Thus, both 8bp repeats appear to be involved in regulation of basal transcription. Further mutation studies eliminated the ATF, c-myc, and MLTF binding sites from consideration and determined that BRCA2 basal transcription is predominantly regulated through the USF binding site.

USF regulates BRCA2 basal transcription – The -34 to -15 region has recently been reported to be responsible for regulation of the basal activity of the BRCA2 promoter (15). The USF binding site was shown to regulate promoter activity in a cell cycle dependent manner, with binding of USF resulting in 3-fold induction of luciferase activity. In addition, the Elf-1 transcription

factor was shown to bind to the Ets consensus binding site (-61 to -54) and to induce activity 3-fold. In order to verify these observations, we carried out gel shift assays with wildtype and mutant probes from the -34 to -15 region. Four oligonucleotide probes, as shown in Table 1, were synthesized and used for gel shift assays with MCF-7 total cell extracts. A specific DNA-protein complex was detected with wild type probe (Figure 2B). The complex binds to repeat 2 and is ablated by mutant forms of this 8bp sequence. The remaining complexes bind to all probes and most likely represent non-specific binding. These findings were further confirmed by competition experiments. A 90-fold excess of unlabeled wild type oligonucleotide probe effectively blocked binding of labeled probe to the protein complex, but had little effect on the non-specific complexes (Figure 2C). To verify that this protein complex contained a member of the USF transcription factor family, as previously suggested, super shift assays were performed using specific antibody against USF1. USF1 antibody efficiently supershifted the complexes formed with the wild type and M-1 DNA probes. In comparison, antibodies against c-myc and ATF failed to supershift the complex (Figure 2D). The combined data strongly suggest that USF binds to the BRCA2 promoter.

In order to address the role of USF in regulation of BRCA2 basal transcription, a series of expression assays were performed. Co-transfection of USF1 or USF2 expression constructs with the Del-15 luciferase reporter construct had no significant effect on luciferase activity in MCF-7 (data not shown). As a control, CREB, c-myc, and c-myc plus max expression constructs were also cotransfected with the reporter constructs into the various cell lines. Ectopic expression of these transcription factors failed to induce luciferase activity (data not shown). Recent studies of USF dependent promoters containing USF consensus binding sites in a variety of cell lines have determined that USF cooperates with transactivating factors to induce expression. In fact, ectopic expression of USF1 or USF2 in most epithelial tumor cell lines, such as MCF-7, results in mini-

mal induction of USF dependent promoters (28). However, in normal mammary epithelial cell lines such as HMEC's (human mammary epithelial cells) (Clonetics) and MCF10A, and in the Saos-2 osteosarcoma cell line, ectopic expression of USF1 or USF2 induced a substantial increase in reporter gene expression and activity (28,29), suggesting a requirement for a cell line specific transactivating factor. To evaluate whether USF must interact with a transactivating partner to induce the BRCA2 promoter, we ectopically expressed a USF-VP16 fusion construct in MCF-7 cells. As shown in Figure 2E, the USF-VP16 fusion protein induced a 4-fold increase in luciferase activity from the full length BRCA2 promoter and from the minimal promoter (Del-15). In addition, mutations in the repeat 2 USF binding site ablated the increased luciferase activity. This suggests that USF interacts with other transactivating proteins to regulate basal transcription from the BRCA2 promoter.

Induction of the BRCA2 promoter by NFκB - Following validation of the role of USF in regulation of BRCA2 basal transcription, we began to systematically map other transcription factor binding sites within the BRCA2 promoter which contribute to regulation of the promoter. Initially, we focused on the -144 to -59 region that was shown to induce basal transcription 3-fold. Sequence analysis of this region identified several putative transcription factor binding sites including an NFκB consensus binding site located at position -116 to -107 in the 8 kb BRCA2 promoter. To examine the role of NFκB in regulation of the BRCA2 promoter, the effect of overexpression of NFκB on luciferase activity was studied. Co-transfection of expression constructs of the p65 and p50 subunits of NFκB with the pGL3Prom reporter construct containing the wild type BRCA2 promoter resulted in significant induction of luciferase activity. Expression of p65 alone and in combination with p50 increased activity 9 and 16-fold respectively (Figure

3A). However, expression of p50 alone resulted in a small reduction in activity in comparison to a vector control.

To determine whether this NFκB site was required for regulation of BRCA2 basal transcription, the consensus GGAATTTCCT site was substituted by TAACTTTCCT in the Del-14 BRCA2 promoter reporter construct. The Del-14 construct and the Del-14 mutant construct were transfected into MCF-7 cells and the luciferase activity was measured as before. As shown in Figure 3B, expression of p65 or p65 with p50 induced a 3 to 6-fold increase in luciferase activity from the wildtype Del-14 promoter in MCF-7 cells, but had little activating effect on the mutant promoter. These data suggest that the NFκB p65 subunit can induce BRCA2 promoter activity by forming a heterodimer with endogeneous or ectopically expressed p50.

NFκB binds to BRCA2 promoter- To determine whether NFκB subunit proteins bind to the NFκB site in the BRCA2 promoter, we performed gel shift assays of MCF-7 whole cell protein extracts with wildtype (WT-κB) and mutant (MT-κB) oligonucleotide probes containing the NFκB site from the BRCA2 promoter (Table 2). Whole cell extracts were prepared from MCF-7 cells 48 hours after transfection with pcDNA 3.1 vector, and NFκB p65 plus p50 expression constructs. Gel shift analysis demonstrated that a protein complex specifically binds to the wildtype NFκB probe, but not to the mutant probe following overexpression of p50 and p65 (Figure 3C). No significant complex formed in the absence of overexpression of these genes. Addition of 100-fold excess of cold competitor DNA probe completely eliminated protein binding to labeled DNA probe (data not shown), suggesting that the protein complex binds specifically to the NFκB site in the BRCA2 promoter. The complex was also supershifted by anti p50 antibody indicating that the NFκB p50 subunit formed part of the complex (Figure 3C). While an anti p65 antibody

did not supershift the complex, a significant decrease in the amount of labeled complex was observed (Figure 3C). Thus, the anti p65 antibody may be binding to p65 in the complex, resulting in reduced access of the DNA probe to the p50 DNA binding subunit of NFκB. These data suggest that a p50/p65 NFκB heterodimer directly interacts with the NFκB-like site in the BRCA2 promoter resulting in direct induction of the promoter.

In vivo induction of BRCA2 by overexpression of NFκB - In order to demonstrate an in vivo effect of NFκB on BRCA2 promoter function, we studied the effect of overexpression of p50 and p65 NFκB subunits on endogeneous BRCA2 expression. As before, p50 and p65 constructs were transfected into MCF-7 cells, and Northern blots of RNA from the cells were hybridized with a full length BRCA2 cDNA probe. Substantial increases in BRCA2 mRNA expression were observed following ectopic expression of p65, and p50 plus p65 (Figure 4A). In addition, western blots of whole cell extracts were hybridized with anti p50 and anti p65 antibodies (Santa Cruz Biotech) to verify expression of the NFκB subunits, and with 9D3 anti BRCA2 antibody (GeneTex) to determine protein levels of BRCA2 in response to expression of NFκB subunits. Transfections of MCF7 cells with p65, and p50 plus p65 constructs resulted in substantially increased levels of these proteins (Figure 4B). BRCA2 protein levels were also significantly increased in response to p65, and p50 plus p65 expression, while BRCA2 levels remained low in vector control transfected cells (Figure 4B). This result verifies that NFκB expression results in induction of BRCA2 expression.

Dominant negative and wildtype IκBα inhibit NFκB dependent induction of BRCA2- To further demonstrate the role of NFκB in regulation of the BRCA2 promoter, the effect of signaling from

the NFκB signaling pathway on BRCA2 promoter induction was assessed. In this study, transfection with a wildtype IκBα or a dominant negative mutant IκBα expression construct was used to block signaling through the NFκB pathway. The dominant negative IκBα mutant (IκBα 32A/36A) (30) is mutated at two phosphorylation sites and cannot be degraded following IKK dependent phosphorylation, resulting in retention of NFκB in the cytoplasm. MCF-7 cells were cotransfected with expression constructs for p65, dn-IκBα, IκBα, p65 plus dn-IκBα, p65 plus IκBα, and vector controls along with the pGL3Prom reporter construct. Expression of dn-IκBα or IκBα in combination with p65 resulted in a significant reduction in luciferase activity when compared to the effect of p65 alone, as shown in Figure 5A. In addition, quantitative RT-PCR analysis of RNA from these transfected cells demonstrated that ectopic expression of dn-IκBα or IκBα significantly reduced the level of expression of BRCA2 (Figure 5B). These data suggest that inhibition of nuclear translocation of NFκB by dn-IκBα or IκBα substantially inhibits BRCA2 promoter activity.

DISCUSSION

Evidence for involvement of BRCA2 in regulation of cellular response to DNA damage (9,12), in cell proliferation (8), in cell cycle regulation (18), and in transcriptional regulation (31-33) has been accumulating. The variety of functions of BRCA2 suggests that regulation of expression levels of this gene may play an important role in regulation of a number of important cellular processes, and that alterations in BRCA2 expression may contribute to tumorigenesis. Interestingly, while no somatic mutations have been identified in BRCA2, apparent overexpression of BRCA2 has been detected in a significant proportion of sporadic breast cancers.

In this study, we have shown that the NFκB transcription factor binds to the BRCA2 promoter and induces expression of the BRCA2 gene. Deletion mapping of the promoter determined that the -144 and -59 region, which contains an NFκB binding site (GGAATTTCCT), is associated with 3 fold activation of the promoter. A combination of gel shift and supershift assays confirmed that NFκB binds to the NFκB cis-element. In addition, ectopic expression of NFκB subunits p65 or p65 plus p50 resulted in induction of the BRCA2 promoter and increased levels of BRCA2 mRNA and protein within MCF-7 cells, while substitution mutations in the NFκB binding site ablated these effects. These data strongly suggest that NFκB can activate the BRCA2 promoter and induce increased expression of the BRCA2 gene.

The NFκB transcription factor consists mostly of p50/p65 heterodimers, which are complexed to IκBα in the cytoplasm of unstimulated cells. Upon activation of the NFκB signaling pathway, degradation of IκBα exposes nuclear localization signals on the p50/p65 heterodimer leading to nuclear translocation, and transcriptional activation of a number of promoters. In this study, we have shown that overexpression of the p50 DNA binding domain of NFκB does not result in upregulation of the BRCA2 promoter. Ectopically expressed p50 most likely forms a heterodimer with endogeneous p65, but as p65 levels are low and the NFκB nuclear localization signals are present in p65, relatively little heterodimer translocates to the nucleus and binds to the BRCA2 promoter. Conversely, expression of the p65 subunit with or without ectopic p50 significantly induced luciferase activity, suggesting that the transactivating p65 subunit is necessary for induction of the BRCA2 promoter. Overexpressed p65 most likely binds to endogeneous p50, saturates IκB, and translocates to the nucleus resulting in upregulation of the BRCA2 promoter. In this case, endogeneous levels of p50 appear to be sufficient to facilitate increased binding of the p50/p65 heterodimer to the promoter. While only the p50 and p65 NFκB subunits were ana-

lyzed in this study, it is likely that the other subunits such as c-Rel, p52, and RelB are also capable of contributing to induction of the BRCA2 promoter.

To verify the role of NFκB in BRCA2 transcriptional regulation we also evaluated the effect of the NFκB signaling pathway on BRCA2 expression. IκB is a component of the NFκB signaling pathway which binds to NFκB and prevents nuclear translocation of NFκB. Thus, overexpression of IκBα, or a dominant negative form of IκBα, which is resistant to IKK dependent degradation, is expected to inhibit NFκB nuclear translocation and NFκB dependent promoter induction. In this study, ectopic expression of both dn-IκBα and IκBα abrogated NFκB dependent BRCA2 promoter induction and downregulated BRCA2 mRNA levels, suggesting that expression of the BRCA2 tumor suppressor can be regulated by modulation of the NFκB signaling pathway.

NFκB is known to regulate expression of a large number of genes that play critical roles in regulation of apoptosis, tumorigenesis and inflamation. In breast cancers, alterations in DNA binding activity, gene expression, and/or nuclear translocation of NFκB proteins have been observed. More specifically, increased NFκB DNA binding activity has been correlated with expression of the c-erbB-2 gene (34), and high levels of NFκB/Rel binding have been observed in carcinogen-induced primary rat mammary tumors (35). As NFκB appears to regulate BRCA2 expression, it seems likely that alterations in NFκB expression and DNA binding (34,35) contribute to the observed overexpression of BRCA2 in breast tumors (26). Thus, alteration of expression of the BRCA2 tumor suppressor gene may be one mechanism by which aberrantly regulated NFκB contributes to tumorigenesis.

Interestingly, a threefold difference in luciferase activity between the pGL3Prom construct and the Del-14 construct was detected in the presence of ectopically expressed p65. This

result suggests that other elements within the BRCA2 promoter are directly or indirectly responsive to NFκB. One other NFκB consensus binding site (GAGAAACCCC) was identified in the promoter at position -808 to -799. However, using deletion constructs and by overexpressing p65, we have shown that this NFκB site does not play a role in regulation of the BRCA2 promoter (data not shown). Thus, other cis-elements that are indirectly affected by NFκB may contribute to regulation of the BRCA2 promoter.

In addition to NFκB responsive elements, we have also identified another activation domain that results in 3-fold reduction in activity when removed, and a single repression domain that results in 2.5-fold activation when removed (Figure 1B). The transcription factor binding sites from these regions and the associated transcription factors that contribute to regulation of the BRCA2 promoter are not yet known. Recently a repression domain associated with 10-fold down regulation of the BRCA2 promoter was reported (36). This domain is associated with two Alu repeats and is located in the Del-7 and Del-8 clones and is deleted from the Del-9 clone shown in Figure1. However, in the current study the repression domain in the BRCA2 promoter maps to a different location (Del-9, Figure 1). Further studies are needed in order to explain the differing results from the two studies.

The role of the USF transcription factor in regulation of basal transcription from the BRCA2 promoter was also verified in the course of this study. A critical 20 bp regulatory sequence (-34 to -15), which is predominantly controlled by binding of USF, and is responsible for the majority of BRCA2 transcription, was identified. The critical 20 bp region contains a tandem repeat sequence (GCGTCACG) (Figure 2A) and consensus DNA binding motifs for transcription factors such as c-myc, ATF, and USF. Gel shift, supershift, and co-transfection studies demonstrated that only USF binds to the second repeat and regulates the BRCA2 promoter. Recently, Davis and colleagues reported that USF binds to the BRCA2 promoter as a heterodimeric

complex of USF-1 and USF-2, and regulates basal transcription in a cell cycle dependent manner (15). In this earlier study, overexpression of USF in MCF-7 cells induced only a 2.5 fold increase in promoter activity. However, the addition of IE62, a varicella zoster viral protein that binds to USF proteins (37), resulted in 12 fold induction of promoter activity. Similarly, we have demonstrated that expression of a USF-VP16 fusion protein enhanced induction of the promoter. These results suggest that a co-activating factor is needed for USF activation of the BRCA2 promoter.

The USF family of basic helix-loop-helix (HLH)-leucine zipper (zip) transcription factors were originally named as major late transcription factors (MLTF) because of their involvement in transcription from the adenovirus major later promoter (38). It is noteworthy that many USF target genes such as p53 (39), transforming growth factor β2 (40) and cyclin B1 (41) are involved in regulation of proliferation and the cell cycle. Moreover, USF overexpression significantly inhibits c-myc-dependent cell transformation (42) and proliferation of certain transformed cells (29). Thus, the activation of the BRCA2 tumor suppressor gene promoter by USF is consistent with the anti-proliferative effect of this transcription factor. The observation that USF transcriptional activity is lost in breast cancer cell lines but not in normal breast epithelial cells (28) further supports a role for USF as a key regulator of breast cancer development. The combination of these studies and our data suggests that regulation of BRCA2 promoter activity by USF may serve an essential role in the prevention of breast cancer development.

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REFERENCES

1. Wooster, R., Bignell, G., Lancaster, J., Swift, S., Seal, S., Mangion, J., Collins, N., Gregory, S., Gumbs, C., Micklem, G., Barfoot, R., Hamoundi, R., Patel, S., Rice, C., Biggs, P., Hashim, Y., Smith, A., Connor, F., Arason, A., Gudmundsson, J., Ficence, D., Kelsell, D., Ford, D., Tonin, P., Bishop, D. T., Spurr, N. K., Ponder, B. A. J., Eeles, R., Peto, J., Devilee, P., Cornelisse, C., Lynch, H., Narod, S., Lenoir, G., Egilsson, V., Barkadottir, R. B., Easton, D. F., Bentley, D. R., Futreal, P. A., Ashworth, A., and Stratton, M. R. (1995) *Nature* **378**(6559), 789-92

2. Tavtigian, S. V., Simard, J., Rommens, J., Couch, F., Shattuck-Eidens, D., Neuhausen, S., Merajver, S., Thorlacius, S., Offit, K., Stoppa-Lyonnet, D., Belanger, C., Bell, R., Berry, S., Bogden, R., Chen, Q., Davis, T., Dumont, M., Frye, C., Hattier, T., Jammulapati, S., Janecki, T., Jiang, P., Kehrer, R., Leblanc, J. F., Mitchell, J. T., McArthur-Morrison, J., Nguyen, K., Peng, Y., Samson, C., Schroeder, M., Snyder, S. C., Steel, L., Stringfellow, M., Stroup, C., Swedlund, B., Swenson, J., Teng, D., Thomas, A., Tran, T., Tran, T., Tranchant, M., Weaver-Feldhaus, J., Wong, A. K. C., Shizuya, H., Eyfjord, J. E., Cannon-Albright, L., Labrie, F., Skolnick, M. H., Weber, B., Kamb, A., and Goldgar, D. E. (1996) *Nat. Genet.* **12**(3), 333-7

3. Thorlacius, S., Struewing, J. P., Hartge, P., Olafsdottir, G. H., Sigvaldason, H., Tryggvadottir, L., Wacholder, S., Tulinius, H., and Eyfjord, J. E. (1998) *Lancet* **352**(9137), 1337-9

4. Easton, D. (1997) *Nat. Genet.* **16**(3), 210-1

5. Bertwistle, D., Swift, S., Marston, N. J., Jackson, L. E., Crossland, S., Crompton, M. R., Marshall, C. J., and Ashworth, A. (1997) *Cancer Res.* **57**(24), 5485-8

6. Mizuta, R., LaSalle, J. M., Cheng, H. L., Shinohara, A., Ogawa, H., Copeland, N., Jenkins, N. A., Lalande, M., and Alt, F. W. (1997) *Proc. Natl. Acad. Sci. USA* **94**(13), 6927-32

7. Wong, A. K. C., Pero, R., Ormonde, P. A., Tavtigian, S. V., and Bartel, P. L. (1997) *J. Biol. Chem.* **272**(51), 31941-4

8. Ludwig, T., Chapman, D. L., Papaioannou, V. E., and Efstratiadis, A. (1997) *Genes & Development* **11**(10), 1226-41

9. Sharan, S. K., Morimatsu, M., Albrecht, U., Lim, D. S., Regel, E., Dinh, C., Sands, A., Eichele, G., Hasty, P., and Bradley, A. (1997) *Nature* **386**(6627), 804-10

10. Connor, F., Bertwistle, D., Mee, P. J., Ross, G. M., Swift, S., Grigorieva, E., Tybulewicz, V. L., and Ashworth, A. (1997) *Nat. Genet.* **17**(4), 423-30

11. Patel, K. J., Vu, V. P., Lee, H., Corcoran, A., Thistlethwaite, F. C., Evans, M. J., Colledge, W. H., Friedman, L. S., Ponder, B. A., and Venkitaraman, A. R. (1998) *Molecular Cell* **1**(3), 347-57

12. Chen, P. L., Chen, C. F., Chen, Y., Xiao, J., Sharp, Z. D., and Lee, W. H. (1998) *Proc. Natl. Acad. Sci. USA* **95**(9), 5287-92

13. Yuan, S. S., Lee, S. Y., Chen, G., Song, M., Tomlinson, G. E., and Lee, E. Y. (1999) *Cancer Res.* **59**(15), 3547-51

14. Vaughn, J. P., Cirisano, F. D., Huper, G., Berchuck, A., Futreal, P. A., Marks, J. R., and Iglehart, J. D. (1996) *Cancer Res.* **56**(20), 4590-4

15. Davis, P. L., Miron, A., Andersen, L. M., Iglehart, J. D., and Marks, J. R. (1999) *Oncogene* **18**(44), 6000-12

16. Spillman, M. A., and Bowcock, A. M. (1996) *Oncogene* **13**(8), 1639-45

17. Rajan, J. V., Marquis, S. T., Gardner, H. P., and Chodosh, L. A. (1997) *Dev. Biol. (Orlando)* **184**(2), 385-401

18. Lee, H., Trainer, A. H., Friedman, L. S., Thistlethwaite, F. C., Evans, M. J., Ponder, B. A., and Venkitaraman, A. R. (1999) *Molecular Cell* **4**(1), 1-10

19. Gretarsdottir, S., Thorlacius, S., Valgardsdottir, R., Gudlaugsdottir, S., Sigurdsson, S., Steinarsdottir, M., Jonasson, J. G., Anamthawat-Jonsson, K., and Eyfjord, J. E. (1998) *Cancer Res.* **58**(5), 859-62

20. Hamann, U., Herbold, C., Costa, S., Solomayer, E. F., Kaufmann, M., Bastert, G., Ulmer, H. U., Frenzel, H., and Komitowski, D. (1996) *Cancer Res.* **56**(9), 1988-90

21. Kerangueven, F., Allione, F., Noguchi, T., Adelaide, J., Sobol, H., Jacquemier, J., and Birnbaum, D. (1995) *Genes, Chromosomes & Cancer* **13**(4), 291-4

22. Bieche, I., Nogues, C., Rivoilan, S., Khodja, A., Latil, A., and Lidereau, R. (1997) *Br. J. Cancer* **76**(11), 1416-8

23. Teng, D. H., Bogden, R., Mitchell, J., Baumgard, M., Bell, R., Berry, S., Davis, T., Ha, P. C., Kehrer, R., Jammulapati, S., Chen, Q., Offit, K., Skolnick, M. H., Tavtigian, S. V., Jhanwar, S., Swedlund, B., Wong, A. K., and Kamb, A. (1996) *Nat. Genet.* **13**(2), 241-4

24. Lancaster, J. M., Wooster, R., Mangion, J., Phelan, C. M., Cochran, C., Gumbs, C., Seal, S., Barfoot, R., Collins, N., Bignell, G., Patel, S., Hamoudi, R., Larsson, C., Wiseman, R. W., Berchuck, A., Iglehart, J. D., Marks, J. R., Ashworth, A., Stratton, M. R., and Futreal, P. A. (1996) *Nat. Genet.* **13**(2), 238-40

25. Collins, N., Wooster, R., and Stratton, M. R. (1997) *Br. J. Cancer* **76**(9), 1150-6

26. Bieche, I., Nogues, C., and Lidereau, R. (1999) *Oncogene* **18**(37), 5232-8

27. Couch, F. J., Rommens, J. M., Neuhausen, S. L., Belanger, C., Dumont, M., Abel, K., Bell, R., Berry, S., Bogden, R., Cannon-Albright, L., Farid, L., Frye, C., Hattier, T., Janecki, T., Jiang, P., Kehrer, R., Leblanc, J. F., McArthur-Morrison, J., Meney, D., Miki, Y., Peng, Y., Samson, C., Schroeder, M., Snyder, S. C., Stringfellow, M., Stroup, C., Swedlund, B., Swensen, J., Teng, D., Thakur, S., Tran, T., Tranchant , M., Welver-Feldhaus, J., Wong, A. K. C., Shizuya, H., Labrie, F., Skolnick, M. H., Goldgar, D. E., Kamb, A., Weber, B. L., Tavtigian, S. V., and Simard, J. (1996) *Genomics* **36**(1), 86-99

28. Ismail, P. M., Lu, T., and Sawadogo, M. (1999) *Oncogene* **18**(40), 5582-91

29. Qyang, Y., Luo, X., Lu, T., Ismail, P. M., Krylov, D., Vinson, C., and Sawadogo, M. (1999) *Mol. Cell. Biol.* **19**(2), 1508-17

30. DiDonato, J., Mercurio, F., Rosette, C., Wu-Li, J., Suyang, H., Ghosh, S., and Karin, M. (1996) *Mol. Cell. Biol.* **16**(4), 1295-304

31. Milner, J., Ponder, B., Hughes-Davies, L., Seltmann, M., and Kouzarides, T. (1997) *Nature* **386**(6627), 772-3

32. Siddique, H., Zou, J. P., Rao, V. N., and Reddy, E. S. (1998) *Oncogene* **16**(17), 2283-5

33. Marmorstein, L. Y., Ouchi, T., and Aaronson, S. A. (1998) *Proc. Natl. Acad. Sci. USA* **95**(23), 13869-74

34. Raziuddin, A., Court, D., Sarkar, F. H., Liu, Y. L., Kung, H., and Raziuddin, R. (1997) *J. Biol. Chem.* **272**(25), 15715-20

35. Sovak, M. A., Bellas, R. E., Kim, D. W., Zanieski, G. J., Rogers, A. E., Traish, A. M., and Sonenshein, G. E. (1997) *J. Clin. Invest.* **100**(12), 2952-60

36. Sharan, C., Hamilton, N. M., Parl, A. K., Singh, P. K., and Chaudhuri, G. (1999) *Biochem. Biophys. Res. Commun.* **265**(2), 285-90

37. Meier, J. L., Luo, X., Sawadogo, M., and Straus, S. E. (1994) *Mol. Cell. Biol.* **14**(10), 6896-906

38. Sawadogo, M., and Roeder, R. G. (1985) *Cell* **43**(1), 165-75

39. Reisman, D., and Rotter, V. (1993) *Nucleic Acids Res.* **21**(2), 345-50

40. Scholtz, B., Kingsley-Kallesen, M., and Rizzino, A. (1996) *J. Biol. Chem.* **271**(50), 32375-80

41. Cogswell, J. P., Godlevski, M. M., Bonham, M., Bisi, J., and Babiss, L. (1995) *Mol. Cell. Biol.* **15**(5), 2782-90

42. Luo, X., and Sawadogo, M. (1996) *Mol. Cell. Biol.* **16**(4), 1367-75

FOOTNOTES

The abbreviations used are: USF, upstream stimulatory factor; NFκB, nuclear factor-κB; IκBα, Inhibitor of κB; MEFs, mouse embryo fibroblasts; BCS, bovine calf serum; PCR, polymerase chain reaction; ATF, activating transcription factor; CREB, CRE-binding protein; MLTF, major late transcription factor; HMEC's, human mammary epithelial cells.

FIGURE LEGENDS

Figure 1. Activity profiles of human BRCA2 promoter luciferase reporter deletion constructs in MCF-7 cells (A) Schematic diagram of an 8 kb fragment of genomic DNA containing the BRCA2 promoter cloned into the pGL3 reporter construct (pGL3Prom). A series of deletion constructs are also shown. The position of the most proximal or distal nucleotide from the

promoter region relative to the transcription start site of BRCA2 is shown for each construct. **(B)** Luciferase activity profiles of the BRCA2 promoter reporter constructs in MCF-7 cells. To control for transfection efficiency cells were cotransfected with pRL-CMV and the activity associated with each construct was normalized relative to Renilla luciferase activity. The luciferase activity for each construct is shown relative to the wildtype pGL3Prom construct.

Figure 2. USF regulates BRCA2 basal transcription (A) The minimal BRCA2 promoter. The minimal promoter (Del-15) contains the region between -58 and +3 relative to the transcription start site. A series of deletion and substitution constructs derived from Del-15 are also shown. Luciferase activities of the BRCA2 promoter reporter constructs in MCF-7 cells relative to the wildtype pGL3Prom construct are indicated. The positions of a tandem repeat 8bp sequence, and putative MLTF, ATF, USF, and c-myc binding sites are indicated. Substituted nucleotides from each mutant construct are underlined. **(B)** A single protein complex binds to the 20bp repeat sequence. Electrophoretic mobility shift assays using wildtype (WT) and mutant (M-1, M-2) oligonucleotide probes (Table 1) and MCF-7 whole cell protein extracts were performed. M-1 contains substitution mutations in the first 8bp repeat and M-2 contains mutations in the second 8bp repeat. The single protein complex is indicated. **(C)** A protein complex binds specifically to the second 8bp repeat. A competition assay was performed using increasing concentrations of unlabeled wildtype oligonucleotide probe. **(D)** The USF transcription factor binds to the second 8bp repeat. Supershift assays were performed with whole cell lysates of MCF-7, anti USF-1, c-myc, and ATF antibodies, and wildtype and mutant oligonucleotides (WT, M-1, M-2). The supershifted complex containing the anti USF antibody and the gel shift complex are indicated. **(E)** USF requires a transactivating cofactor to activate the BRCA2 promoter. MCF7 cells were transfected with pGL3Prom, Del-15, Del-15-1 (substitution in the first repeat), Del-15-2 (substitution

in the second repeat), or Del-15-1+2 (substitutions in both repeats) constructs along with a pCMV-USF-VP16 construct containing a USF and VP16 fusion gene, or a vector control. Luciferase activities were normalized by protein concentration and are shown relative to the activity from the pGL3Prom wildtype construct.

Figure 3. Nuclear factor κB induces the BRCA2 promoter

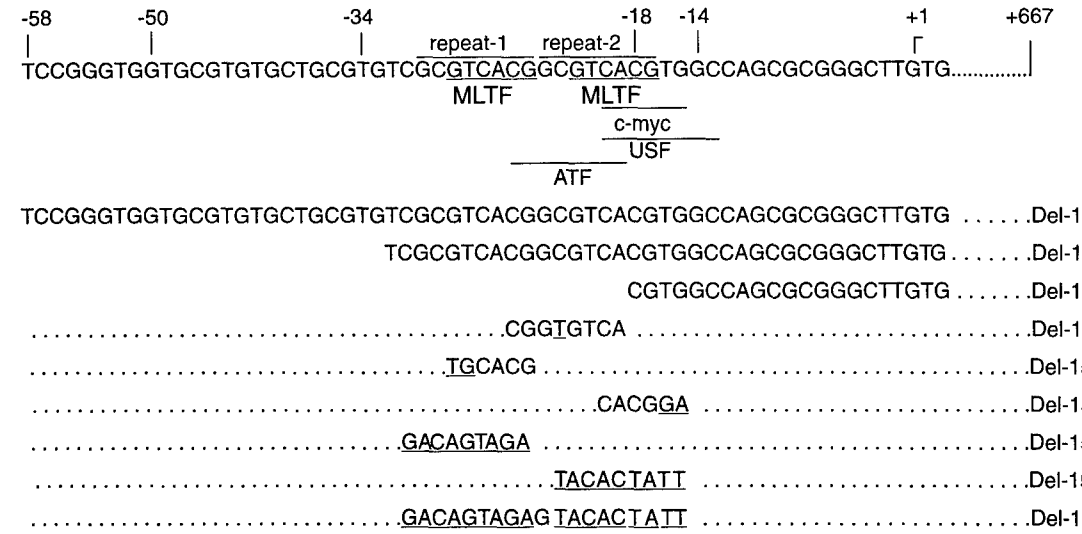
(A) Ectopic expression of the p65 and p50 NFκB subunits activates the pGL3Prom wildtype BRCA2 promoter in MCF-7 cells. The pGL3Prom wild type BRCA2 promoter reporter gene construct was transfected into MCF-7 cells with a pRL-CMV Renilla luciferase construct and either pcDNA 3.1 (vector), p50 expression construct (p50), p65 expression construct (p65), or p50 and p65 expression constructs (p50+p65). Luciferase activities were normalized by the Renilla luciferase activity, and are presented relative to the pcDNA3.1 control. **(B)** Ectopic expression of the p65 and p50 NFκB subunits activates the minimal BRCA2 promoter in MCF-7 cells. The Del-14 wildtype and Del-14 mutant (substitution in the NFκB consensus binding site) reporter constructs were transfected with p50 and p65 expression constructs or a vector control. Luciferase activity was normalized as before and is shown relative to activity from the Del-14 wildtype. **(C)** NFκB p50/p65 heterodimers bind to the NFκB consensus binding site in the BRCA2 promoter in MCF-7 cells. Gel shift assays for the p50 and p65 NFκB subunits with wildtype (W) and mutant (M) oligonucleotide probes for the NFκB consensus binding site in the BRCA2 promoter are shown. Gel shifts were performed using extracts from pcDNA3.1 or p65 and p50 transfected MCF-7 cells. Supershift assays were performed with anti p50 and anti p65 antibodies.

Figure 4. Expression of NFκB subunits results in increased BRCA2 expression

(A) Overexpressed NFκB subunits induce BRCA2 mRNA expression. Total RNA isolated from MCF-7 cells 48 hr after transfection with vector, p65 or p65 plus p50, was Northern blotted with α-^{32}P-ATP labeled human BRCA2 cDNA probe. The level of BRCA2 mRNA was normalized by GAPDH. **(B)** Overexpression of NFκB subunits increase BRCA2 protein levels. Cell lysates isolated from MCF-7 cells 48 hr after transfection with vector, p65, or p65 plus p50 were prepared as described above and used for Western blotting with primary antibodies against BRCA2, p50, p65 or a histone-1 control.

Figure 5. Inhibition of the NFκB signaling pathway prevents induction of the BRCA2 promoter. (A) Wildtype and dominant negative mutant IκBα block NFκB dependent induction of the BRCA2 promoter. MCF-7 cells were cotransfected with IκBα, dn- IκBα, p65, dn- IκBα plus p65, IκBα plus p65, and vector controls along with the pGL3Prom BRCA2 promoter luciferase reporter. After 48hr luciferase activity in whole cell lysates was measured and normalized against Renilla luciferase activity. Luciferase activity relative to the vector control is shown for each transfection. **(B)** Wildtype and dominant negative mutant IκBα inhibit NFκB dependent BRCA2 expression. RNA prepared from the transfections in (A) was used for quantitative RT-PCR of BRCA2. PCR products for BRCA2 and the GAPDH normalization control for each transfection are shown.

2A



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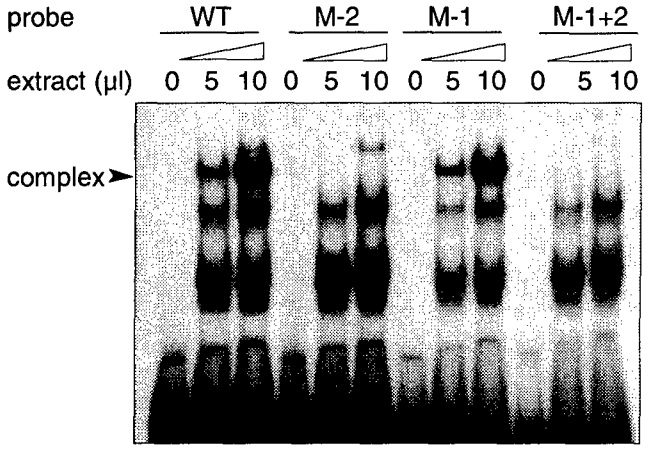

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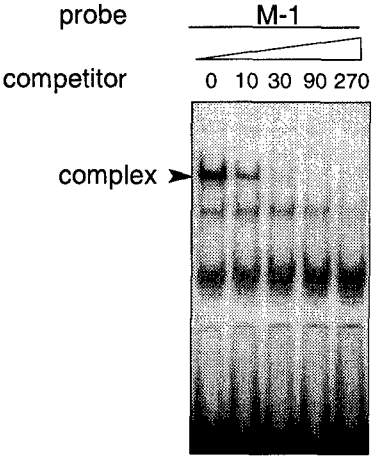

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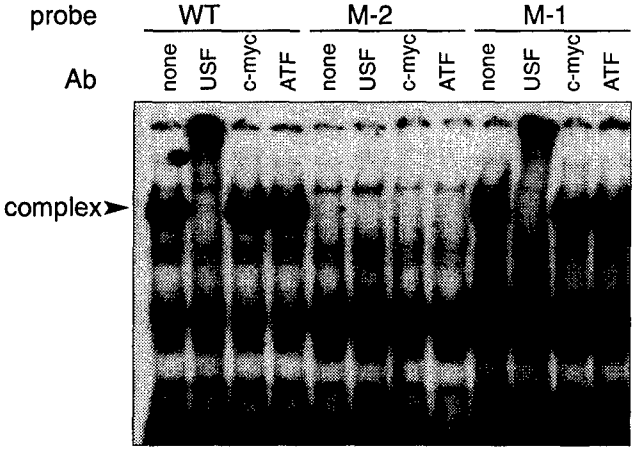

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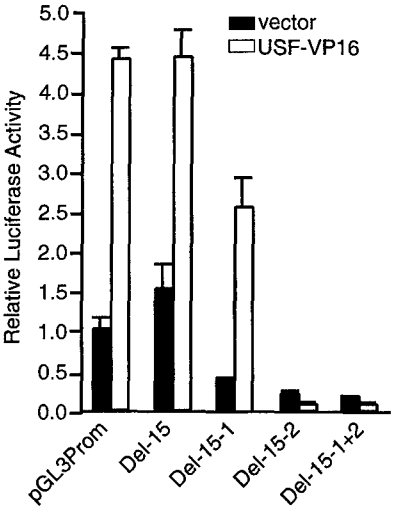
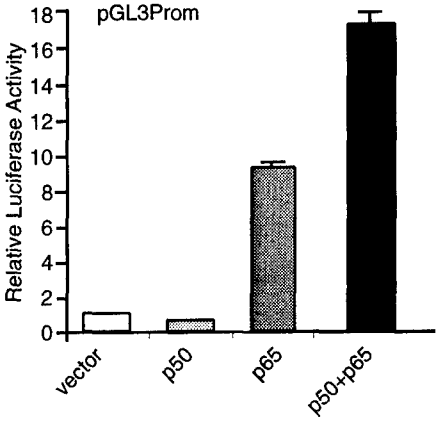


Wu, et al., Fig. 2

3A



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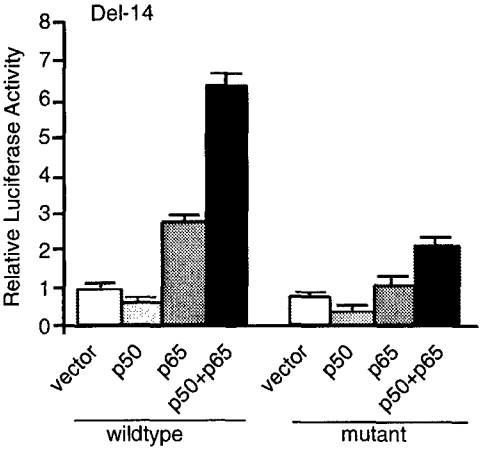

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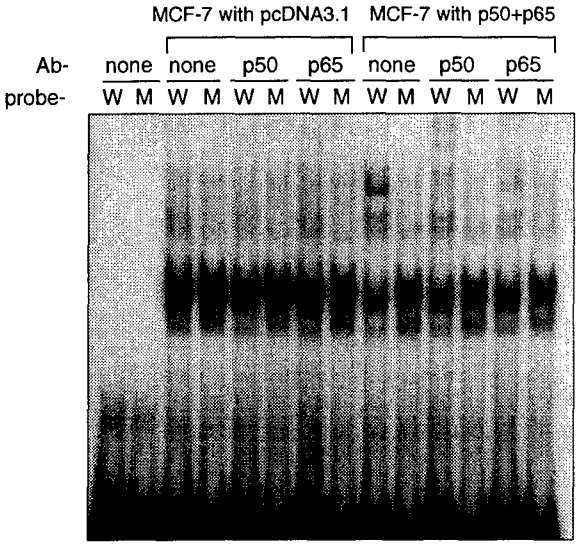
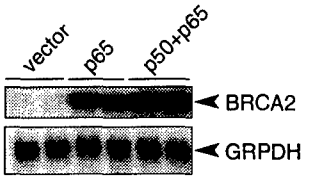


Wu, et al., Fig. 3

4A



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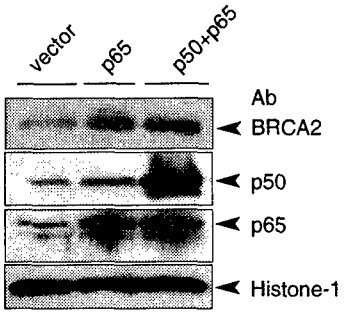

Wu, et al., Fig. 4

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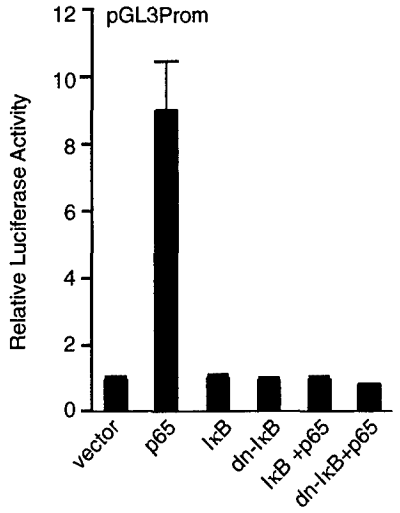
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Relative Luciferase Activity
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8
6
4
2
0
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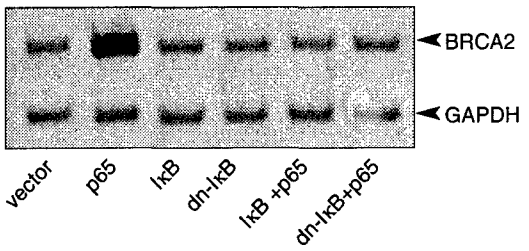
IκB
dn-IκB
IκB +p65
dn-IκB+p65

B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GAPDH
vector
p65
IκB
dn-IκB
IκB +p65
dn-IκB+p65

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REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 21 Feb 03

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2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

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